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Taxonomic studies on *Mucor inaequisporus,* isolated for the first time in South America

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Abstract— *Mucor inaequisporus* (*Mucorales*, *Mucoromycotina*) was isolated from *Syzygium cumini*, a common introduced tree species in Brazil. It is distinguished from similar species by producing straight or undulate sporangiophores with randomly distributed irregular swellings. The columellae are mostly pyriform at 25 °C, but oblong, conic, ellipsoid, and obovoid columellae were also found. The sporangiospores vary in size and shape, with a minor proportion exhibiting an irregular shape. ITS-based phylogenetic analyses reveal that *M. inaequisporus* is closely related to *M. amphibiorum* and *M. nederlandicus*. This species can grow between 20 and 30 °C with optimal development at 25 °C, but variations in the shape of sporangiospores and columellae caused by incubation under different temperatures were observed. No growth was detected at 10 and 40 °C.

Key words— DNA sequencing, growth rate, zygomycete

Introduction

Mucor Fresen., which belongs to the subphylum *Mucoromycotina* (Hibbett et al. 2007), is characterized by the formation of non-apophysate sporangia (with wet or dry walls when mature) on top of simple or branched sporangiophores that emerge from the substrate (Benny 2006). Taxa of this genus have been isolated from soil, dung, decaying fruits, and plants (White et al. 2006, Santiago & Souza-Motta 2008, Santiago et al. 2011), while others are considered the causal agent of cutaneous zygomycosis in humans (Álvarez et al. 2011). Several species are able to produce enzymes with biotechnological applications (Alves et al. 2002a), and some are used to prepare fermented food (Hesseltine 1983, Abe et al. 2004).

Previous studies have treated *Mucor* as polyphyletic and highlighted the difficulty of establishing subgeneric groups (O'Donnell et al. 2001, Voigt & Wöstemeyer 2001, Jacobs & Botha 2008). Although over 300 species are cited in the literature (Jacobs & Botha 2008), the actual number of species may range only from 50 to 75 (Gherbawy et al. 2010). After revising several *Mucor* species, Schipper (1978) reduced the proposed taxa to 39 species, four varieties, and 11 forms. Subsequently, another 17 species have been described as new taxa (Mehrotra & Mehrotra 1979; Mirza et al. 1979, Subrahamanyam 1983, Chen & Zheng 1986, Schipper 1989, Schipper & Samson 1994, Watanabe 1994, Pei 2000, Alves et al. 2002b, Jacobs & Botha 2008, Álvarez et al. 2011, Madden et al. 2012).

Mucor inaequisporus was first described by Dade (1937) from *Spondias mombin* L. fruits in Aburi, Ghana. Since then, a few records of this species from several fruits have been reported. Morphologically, *M. inaequisporus* is mainly characterized by the highly variable form and size of columellae and sporangiospores (Schipper 1978).

The present study describes *Mucor inaequisporus* isolated from a fruit of *Syzygium cumini* (L.) Skeels (= *Eugenia jambolana* Lam.; *Myrtaceae*), commonly found in the State of São Paulo and known locally as 'Jambolão' or 'Jamelão.' We discuss the influence of temperature, light, and culture media on growth and on morphology of *M. inaequisporus*.

Materials and methods

Isolation and morphological identification of *M. inaequisporus*

Mature fruits of *S. cumini* were collected on the campus of the Federal University of São Carlos (21°59′02″S 47°52′55″W), São Paulo, Brazil. In February 2009 (URM 6532) and February 2010 (URM 6533), mycelium fragments were removed directly from growing fruits under a stereomicroscope (Leica EZ4) and transferred to Petri dishes with potato dextrose agar (PDA) (Benny 2008) supplemented with chloramphenicol (Neo Fenicol – Neo Química) (80 mg L^{-1}). Plates were left on a bench at room temperature (28 ± 2 °C) for 7 days in alternating light and dark periods. The strains were subcultured in order to obtain pure cultures. Identification was based on macroscopic (color, aspect and diameter of the colonies) and microscopic characters according to Dade (1937) and Schipper (1978).

Influence of temperature, culture media, and light on the growth and morphology of *M. inaequisporus*

Pure cultures were grown in malt extract agar (MEA) and PDA (Benny 2008) at 15, 20, 25, 30, 35, and 40 °C for 7 days under light and dark periods. Mycelial fragments were removed from cultures to prepare wet mounts using KOH (3%) and studied under bright-field microscopy (BFM) (Carl Zeiss Axioscope 40). Color designation followed Watling (1969). The morphological characteristics were also studied using Environmental Scanning Electron Microscopy (ESEM). Agar blocks retrieved from

Table 1. *Cokeromyces* and *Mucor* species included in the molecular analysis

Species	Isolate	GenBank No.
Cokeromyces recurvatus Poitras	CBS 158.50^{T}	DQ118986
M. amphibiorum Schipper	CBS 763.74^{T}	FJ455861
M. bainieri B.S. Mehrotra & Baijal	CBS 293.63	JF299222
M. circinelloides Tiegh. (as *M. circinelloides* f. *circinelloides*)	CBS 195.68^{NT}	DQ118991
M. ellipsoideus Ed. Álvarez et al.	UTHSC 02-2090^{T}	FN650647
M. flavus Bainier	CBS 230.35	EU484282
M. fragilis Bainier	CBS 236.35	FN650655
M. fuscus Bainier	CBS 132.22^{T}	FN650653
M. genevensis Lendn.	CBS 114.08^{T}	EU484275
M. guilliermondii Nadson & Filippov	CBS 174.27^{T}	JF299231
M. hiemalis f. *corticola* (Hagem) Schipper	CBS 106.09	AY243950
M. hiemalis Wehmer f. *hiemalis*	CBS 201.65	DQ118992
M. inaequisporus	CBS 255.36^{T}	JN206177
	CBS 351.50	JN206178
	URM 6532	JQ014007
M. indicus Lendn.	CBS 226.29^{T}	DQ118994
M. irregularis Stchigel et al.	CBS 103.93^{T}	DQ119006
M. lusitanicus Bruderl. (as *M. circinelloides* f. *lusitanicus*)	CBS 108.17^{T}	FN650644
M. mucedo Fresen.	CBS 109.16	EU484199
M. nederlandicus Váňová	CBS 735.70	JF299219
M. nidicola Madden et al. (as *M. hiemalis*)	CBS 638.67	JF299220
M. piriformis A. Fisch.	CBS 169.25	EU484276
M. plumbeus Bonord.	CBS 111.07	AF412290
M. racemosus Fresen.	CBS 260.68^{T}	DQ118996
M. ramosissimus Samouts.	CBS 135.65^{NT}	FN650643
M. variosporus Schipper	CBS 837.70^{T}	HM623322
M. velutinosus Ed. Álvarez et al.	UTHSC 06-1667	FN650652
M. zonatus Milko	CBS 148.69^{T}	JF299232

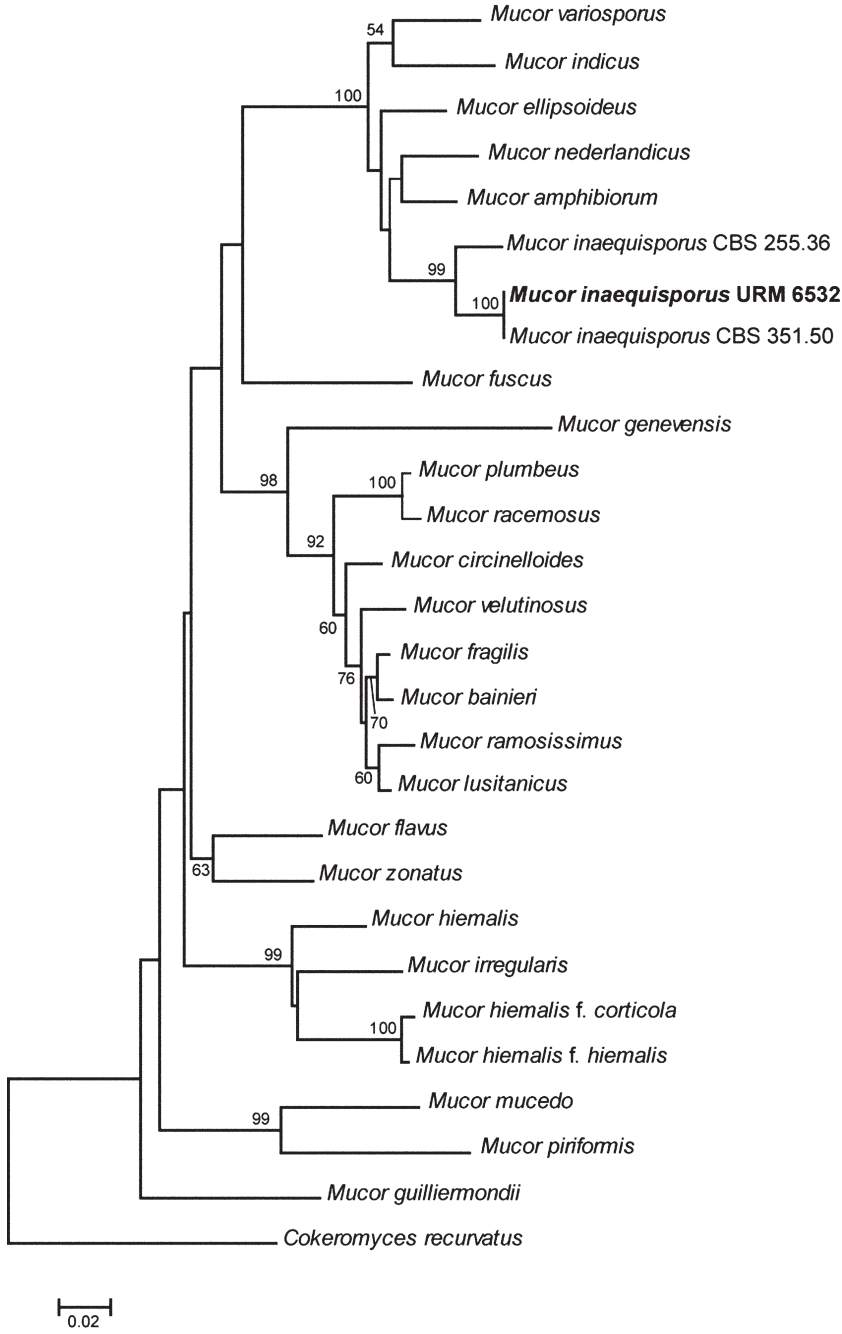
CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; UTHSC = Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA; URM = University Recife Mycology, Recife, Brazil

^{NT} = neotype strain; ^{T} = type strain

cultures were mounted in microscope slides for direct observation with a scanning electron microscope (FEI Quanta™ 250).

Phylogenetic analyses

DNA was extracted following Gerardo et al. (2004). Cultures of *M. inaequisporus* URM 6532 were cultured in malt broth (2%) at 25 °C for seven days and the mycelium was freeze-dried and disrupted using liquid nitrogen. Genomic DNA was recovered using CTAB and purified with 70% ethanol. PCR primer pair ITS4 and ITS5 was used to amplify the ITS1-5.8S rDNA-ITS2 region (White et al. 1990). Both forward and reverse sequences were generated in ABI3500 (Applied Biosystems). Sequences were assembled in Bioedit v. 7.0.5.3 (Hall 1999) and the consensus was aligned with sequences of several *Mucor* species retrieved from GenBank using Muscle v. 3.6 (Edgar 2004) (Table 1). A neighbor-joining tree was inferred in MEGA5 (Tamura et al. 2011) under the


Mucor variosporus
54
Mucor indicus
100
Mucor ellipsoideus
Mucor nederlandicus
Mucor amphibiorum
Mucor inaequisporus CBS 255.36
99
100
Mucor inaequisporus URM 6532
Mucor inaequisporus CBS 351.50
Mucor fuscus
Mucor genevensis
98
100
Mucor plumbeus
Mucor racemosus
92
Mucor circinelloides
Mucor velutinosus
60
Mucor fragilis
76
Mucor bainieri
70
Mucor ramosissimus
60
Mucor lusitanicus
Mucor flavus
63
Mucor zonatus
Mucor hiemalis
99
Mucor irregularis
Mucor hiemalis f. corticola
100
Mucor hiemalis f. hiemalis
Mucor mucedo
99
Mucor piriformis
Mucor guilliermondii
Cokeromyces recurvatus
0.02

maximum-composite likelihood algorithm to calculate genetic distances. Robustness was calculated using 1000 bootstrap pseudo-replicates, and the tree was rooted with *Cokeromyces recurvatus* (Table 1).

Results

Phylogenetic Analysis

The size of the ITS region of *M. inaequisporus* URM 6532 is 536 bp. BLASTn comparisons with sequences deposited at the GenBank revealed that the ITS sequence of this strain is 92% identical with a sequence labeled as *Mucoraceae* LM042 (EF060714), 91% identical with another undescribed *Mucor* species (AB638465), 90% identical to the sequence of *M. amphibiorum* (FJ455861), 95% identical with *M. inaequisporus* (CBS 255.36), and 100% identical with *M. inaequisporus* (CBS 351.50). The phylogenetic relationship among *Mucor* species based on the ITS region is illustrated in Fig. 1. *Mucor guilliermondii* appears as the basal species, phylogenetically distant from other species in the genus. *Mucor inaequisporus* was genetically distant from *M. mucedo*, the type species of the genus, and clustered in a well-supported clade (100% bootstrap value) with *M. nederlandicus*, *M. amphibiorum*, *M. ellipsoideus*, *M. variosporus*, and *M. indicus*. Another well-supported clade (98% bootstrap value) included *M. ramosissimus*, *M. lusitanicus*, *M. bainieri*, *M. fragilis*, *M. circinelloides*, *M. velutinosus*, *M. plumbeus*, *M. racemosus*, and *M. genevensis.* Phylogenetic analysis indicates only distant relatedness within two pairs of morphologically similar species, *M. genevensis*/*M. hiemalis* and *M. nederlandicus*/*M. guilliermondii*.

Taxonomy

Mucor inaequisporus Dade, Trans. Br. Mycol. Soc. 21(1): 25. 1937. Pl. 1

Colonies grow rapidly on MEA, filling the entire Petri dish (9 cm diam. and 10 mm height) after 72 hours at 25 °C in the dark. The colony ranges from an obverse intense yellow (8G to luteus 51) with yellow to olive-gray spots, corresponding to sporangia, and reverse yellow (8G), particularly at the point of inoculum. Rhizoids were short to long (≤600 μm in length), poorly branched, with or without septa. A sweet aroma was produced. Sporangiophores 9–30 μm in diam., with or without yellow droplets, simple or with long and short sympodial branches (3–10 times, mostly after 6 days of incubation), erect, undulant, curved, sometimes with one or several randomly spaced septa and sometimes constricted next to the sporangia; phototropic,

Fig. 1. Neighbor-joining tree of *Mucor* spp. inferred from the ITS-rDNA region. The maximum-composite likelihood algorithm was used to calculate genetic distances. Numbers on branches are bootstrap support values obtained from 1000 pseudo-replicates (values under 50% are not shown). A sequence of *Cokeromyces recurvatus* was used to root the tree. *Mucor inaequisporus* examined in this work is denoted in bold. Scale bar indicates the number of base substitutions per site.

with yellow to chestnut contents and with incrusted walls. Some sporophores exhibit randomly distributed irregular swellings (≤47 µm in diam.), frequently gradually tapering towards the base. Sterile branches are common. Sporangia globose and subglobose to lightly depressed, yellow and turn brown with age, with finely echinulated, transparent and deliquescent walls that are more persistent in the smaller sporangia, (47.5–)70–130(–180) × (45–)60–120 (–165) µm; Columellae highly variable in shape, frequently pyriform (mostly in the larger ones), conic or oblong, but also ellipsoid, obovoid, subglobose, colorless or slightly yellow to chestnut; smooth walled with prominent collars, (45–)60–100(–140) × (35–)50–80(–120) µm; Sporangiospores hyaline with granular contents, highly variable in shape and size: majority elliptical (sometimes flattened at one side), 5–14(–22.5) × 3.5–10(–15) µm, also globose, subglobose (≤15 µm diam.), some irregular (≤24 × 15 µm). Chlamydospores and zygosporangia not observed.

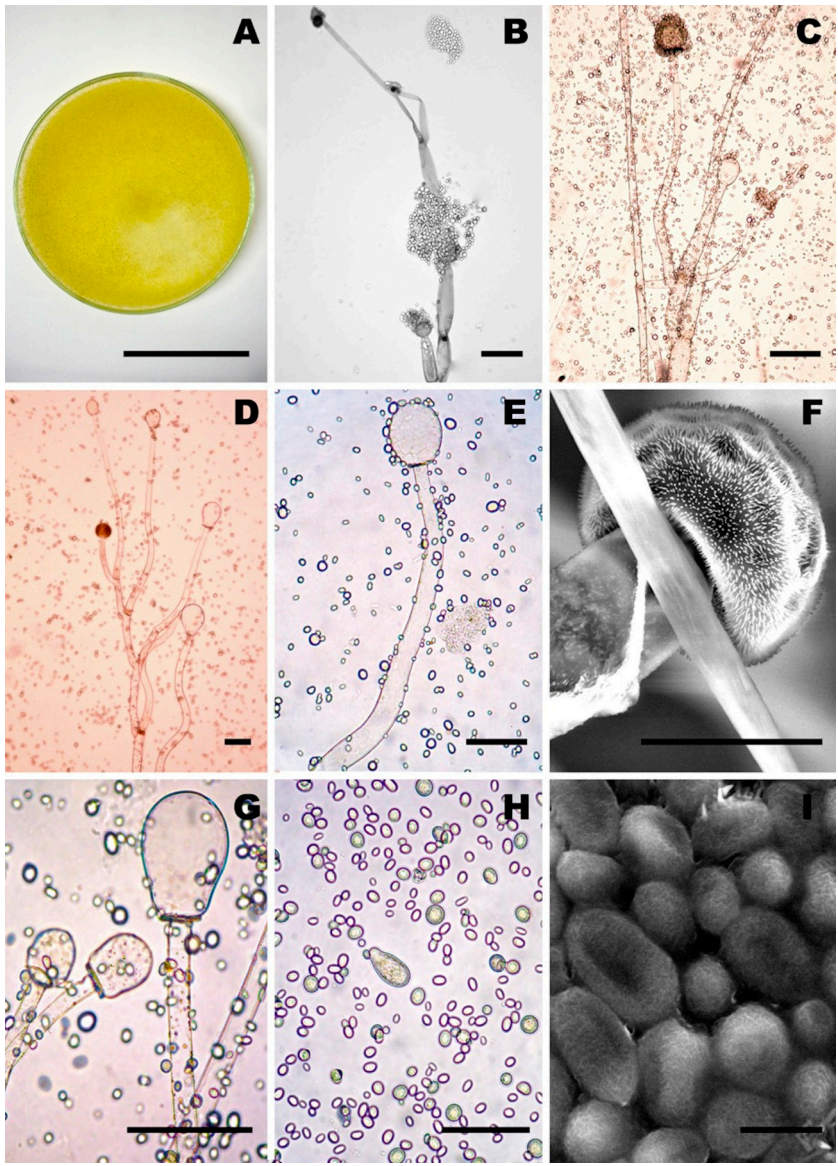
Influence of temperature, culture media and light on the growth and morphology of *M. inaequisporus*

Macroscopic and microscopic features on PDA were similar to those on MEA. The optimum growth temperature was 25 °C (colonies filled the entire Petri plate after 72 hours) with good growth and sporulation between 15 and 30 °C. Limited growth was recorded at 35 °C and no growth was observed at 10 and 40 °C. Colonies incubated at 30 °C grew well (9 cm diam. after 72 hours), but were shorter in height (6 mm) and the sporangiophores were more branched than at 25 °C. The columellae were mainly oblong and conical, rarely pyriform, and the sporangiospores were irregular in shape, predominantly globose and subglobose. Colonies incubated at 20 °C reached 9 cm in diam. after 72 hours. Such colonies were irregularly zonate, at raised in the center and then touching the entire lid of the Petri dish after 7 days. Abundant twisted sterile and coiled hyphae were observed in the aerial mycelium. Colonies were also irregularly zonate at 15 °C, reaching 9 cm diam. and 2 mm in height after 96 hours. Abundant twisted sterile hyphae were also observed in the aerial mycelium. Influence of light was not detected.

Specimens examined — **BRAZIL**, **São Paulo**: Campus of the Federal University of São Carlos, (21°59′02″S 47°52′55″W), on mature fruits of *Syzygium cumini*, 23.01.2009, leg. E.M. Canedo (URM 6532); 12.02.2010, leg. E.M. Canedo (URM 6533).

Habitat — Growing on fruits of *Artocarpus glaucus*, *Bouea macrophylla*, *Diospyros kaki*, *Flacourtia inermis*, *Musa paradisiaca*, *M. sapientum*, *Spondias mombin*, *Syzygium cumini*, and *Theobroma cacao* (Dade 1937, Boedijn 1959, CBS Database, IMI Database) and in the rhizosphere of root-knot nematode host plants (Zangeneh et al. 2007).

Distribution — Ghana, Indonesia, Iran, Malaysia, and Brazil. This is the first report for South America.



Pl. 1. *Mucor inaequisporus* grown in MEA at 25 °C for 7 days. A. Colony growing in a Petri dish. B–D. Branched sporangiophores, some with randomly distributed irregular swellings with sporangia and columellae. E. Single undulate sporangiophore with columella. F. Young sporangiophore. G. Columellae. H–I. Sporangiospores. Scale bars. A = 5 cm; B, D–F, H = 50 µm; C, G =100 µm; I = 5 µm. (A–E, G, H = pictures under BFM; F, I = pictures under ESEM.)

Discussion

Several studies have reported the variability of nucleotide sequences of the ITS-rDNA region as a reliable indicator to differentiate taxa of *Mucorales* at the species level, including taxa of *Mucor* (Iwen et al. 2002, Schwarz et al. 2006, Hoffmann et al. 2009). Strain URM 6532 shared an identical ITS sequence with strain CBS 351.50. According to our phylogenetic analysis, *M. inaequisporus* is genetically distinct from other described *Mucor* species. This species exhibited low percentages of similarity with *M. nederlandicus* (91%), with *M. amphibiorum* and *M. variosporus* (90%), and with *M. ellipsoideus* (89%). According to Walther et al. (2013), the ITS is a barcode marker for mucoralean species identification, despite the wide intraspecific variations found for some taxa. *Mucor inaequisporus* appears to have some ITS variability because strain URM 6532 showed a 95% similarity with strain CBS 255.36. Thus, our phylogeny is in agreement with the phylogeny presented in Walther et al. (2013).

Morphologically, *M. inaequisporus* is distinguished from other *Mucor* species by the production of sporophores that are straight, curved, or undulant, and with randomly distributed irregular swellings. The columellae are variable in form, being predominantly pyriform at 25 °C. Sporangiospores are extremely variable in size and shape at 25 °C. However, some features of our isolate, such as curved or undulant sporophores with irregular swellings, were not described by Dade (1937) and Schipper (1978). Columellae sizes reported in the literature (≤100 × 90 µm, Dade 1937; 83–75 µm, Schipper 1978) were smaller than our maximum measurements, whereas sporangiospores (≤25 × 20 µm, Dade 1937; ≤30 × 23 µm, Schipper 1978) were larger than our maximum measurements. According to Schipper (1978), different strains of *M. inaequisporus* may exhibit variable morphologic and physiologic features.

Our results show that *M. inaequisporus* exhibited growth from 15 to 30 °C. However, colonies incubated at 30 °C showed an increased number of branches. The columellae were mostly oblong and conical, rarely pyriform, and the sporangiospores were mostly globose, subglobose, and irregular in shape, different from the observed at 25 °C. Schipper (1975, 1976) has noted the influence of temperature on the shape of columellae and/or sporangiospores in other *Mucor* species such as *M. plasmaticus* Tiegh., *M. psychrophilus* Milko, and *M. racemosus* f. *chibinensis* (Neophyt.).

We report for the first time the occurrence of *M. inaequisporus* in Brazil, thereby expanding the knowledge of *Mucorales* distribution. Strains URM 6532 and URM 6533 were isolated from a common introduced tree species in Brazil, *S. cumini,* which belongs to the *Myrtaceae*. As this is the first report of a *Mucor* species in fruits in this country, this record adds to the twenty-four species of *Mucor* that have been isolated from soil and/or dung in Brazil (Santiago 2012).

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